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May 4, 2001

**BOX PCT**Assistant Commissioner  
for Patents  
Washington, D.C. 20231PCT/GB99/03756 - filed  
November 11, 1999

Re: Application of **Stein O. DØSKELAND**,  
**Margrethe H. SERRES** and  
**Kari E. FLADMARK** entitled "ASSAY FOR  
 PHOSPHATASE-TARGETING TOXINS"  
 Assignee: **BIOSENSE LABORATORIES AS**  
Our Ref: **Q-64374**

Dear Sir:

The following documents and fees are submitted herewith in connection with the above application for the purpose of entering the National stage under 35 U.S.C. § 371 and in accordance with Chapter II of the Patent Cooperation Treaty:

- ☐ an executed Declaration and Power of Attorney.
- ☐ an English translation of the International Application.
- ☒ 4 sheets of drawings.
- ☐ an English translation of Article 19 claim amendments.
- ☒ a Written Opinion.
- ☒ an International Preliminary Examination Report (accompanied by Article 34 amendments attached).
- ☐ an executed Assignment and PTO 1595 form.
- ☐ a Form PTO-1449 listing the ISR references, and a complete copy of each reference.
- ☒ a Preliminary Amendment

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Assistant Commissioner  
for PatentsMay 4, 2001  
Page 2

The Declaration and Power of Attorney, Assignment and Information Disclosure Statement listing the International Search Report and a complete copy of each reference will be submitted at a later date.

It is assumed that copies of the International Application, the International Preliminary Examination Report, and any Articles 19 and 34 amendments as required by § 371(c) will be supplied directly by the International Bureau. However, for the Examiner's convenience, a copy of each of the above-listed documents is submitted herewith.

Priority is claimed from November 11, 1998, based on GB Application No. 9824772.9.

The Assignee for published patent application is **BIOSENSE LABORATORIES AS**.

The Government filing fee is calculated as follows:

Total claims	<u>20</u> - <u>20</u> = _____	x \$18.00 =	<u>\$0.00</u>
Independent			
claims	<u>2</u> - <u>3</u> = _____	x \$80.00 =	<u>\$0.00</u>
Base Fee			<u>\$860.00</u>
<b>TOTAL FEE</b>			<u><b>\$860.00</b></u>

A check for the statutory filing fee, in the amount of \$860.00, is attached.

The Assistant Commissioner is hereby directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880. The Assistant Commissioner is also hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.492 which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Respectfully submitted,

  
Gordon Kit

Registration No. 30,764

09/831108 04 MAY 2001

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Stein O. DØSKELAND et al

CHAPT II of

Appln. No.: PCT/GB99/03756

Group Art Unit: 0000

Filed: May 4, 2001

Examiner: Unknown

For: ASSAY FOR PHOSPHATASE-TARGETING TOXINS  
(As Amended)

PRELIMINARY AMENDMENT

Assistant Commissioner  
of Patents  
Washington, D.C. 20231

Sir:

Prior to examining the above-identified application, please  
amend the application as follows.

IN THE TITLE:

Please delete the present title, and insert therefor

-- ASSAY FOR PHOSPHATASE-TARGETING TOXINS --.

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 1, before line 4, insert

-- This application is a 371 of PCT/GB99/03756, filed  
November 11, 1999. --

PRELIMINARY AMENDMENT  
CHAPT II of PCT/GB99/03756

IN THE CLAIMS:

Please amend the claims as follows:

Claim 3. (Amended) An assay method as claimed in claim 1, wherein the toxin to be determined is a hepatotoxin or okadaic acid.

Claim 4. (Amended) An assay method as claimed in claim 1, wherein toxin molecules present in the sample compete with the non-immobilized ligand for a limited number of binding sites of the immobilized ligand and any toxin present in said sample is determined relative to the extent of non-immobilized ligand bound or not bound to the binding sites of the immobilized ligand.

Claim 5. (Amended) An assay method as claimed in claim 1, wherein the presence or absence of a phosphatase-targeting toxin is determined.

Claim 6. (Amended) An assay method as claimed in claim 1, wherein the sample under investigation is surface or free moisture from shellfish, or water taken from the habitat in which such shellfish live, or water taken from domestic water supplies.

Claim 7. An assay method as claimed in claim 1, wherein the immobilized or non-immobilized ligand is an antibody or antibody fragment.

Claim 8. (Amended) An assay method as claimed in claim 1, wherein the protein phosphatase enzyme is the binding ligand protein phosphatase 2A.

PRELIMINARY AMENDMENT  
CHAPT II of PCT/GB99/03756

Claim 9. (Amended) An assay method as claimed in claim 1, wherein either the immobilized or non-immobilized ligands carries a reporter moiety.

Claim 13. (Amended) An assay method as claimed in claim 1, wherein the solid support is a dipstick or solid matrix.

Claim 17. (Amended) A kit as claimed in claim 15, wherein said kit comprises:

a solid phase upon which is immobilized a protein phosphatase enzyme as a toxin binding ligand;

a reporter molecule capable of competitively inhibiting binding of phosphatase-targeting toxins to said toxin binding ligand and generating a signal readable without laboratory equipment.

Claim 18. (Amended) A kit as claimed in claim 15, wherein said kit comprises magnetically displaceable polymer micro spheres having immobilized thereon a protein phosphatase;

gold sol labeled peptide heaptotoxin molecules capable of competitively inhibiting cyanobacterial toxins binding to said protein phosphatase.

Claim 19. (Amended) A kit as claimed in claim 15, wherein said kit comprises magnetically displaceable polymer micro spheres having immobilized thereon a protein phosphatase;

gold sol labeled okadaic acid molecules capable of competitively inhibiting algal toxins binding to said protein phosphatase.

Claim 20. (Amended) Use of the kit as claimed in claim 15, for the determination of phosphatase-targeting toxins.

PRELIMINARY AMENDMENT  
CHAPT II of PCT/GB99/03756

IN THE ABSTRACT:


Please insert the Abstract attached hereto.

REMARKS

The title has been replaced in order to correct a typographical error, the specification has been amended to insert formal matter, the claims have amended to delete their improper multiply dependency and the Abstract has been inserted in order to make the application consistent with U.S. patent practice. Hence, the replacement of the title, the amendment of the specification and claims and the addition of the Abstract does not constitute new matter.

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,

  
\_\_\_\_\_  
Gordon Kit  
Registration No. 30,764

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Date: May 4, 2001

## A P P E N D I X

### Marked-up Version to Show Changes:

#### IN THE TITLE:

The title has been amended as follows:

ASSAY FOR [PHOSPHATASE-TARGETTING] PHOSPHATASE-TARGETING  
TOXINS.

#### IN THE SPECIFICATION:

The specification is amended as follows:

Page 1, before line 4, the following is inserted:

--- This application is a 371 of PCT/GB99/03756, filed  
November 11, 1999. --.

#### IN THE CLAIMS:

The claims are amended as follows:

Claim 3. (Amended) An assay method as claimed in [either  
of claims 1 and 2] claim 1, wherein the toxin to be determined  
is a hepatotoxin or okadaic acid.

Claim 4. (Amended) An assay method as claimed in [any one  
of claims 1 to 3] claim 1, wherein toxin molecules present in  
the sample compete with the non-immobilized ligand for a limited  
number of binding sites of the immobilized ligand and any toxin  
present in said sample is determined relative to the extent of  
non-immobilized ligand bound or not bound to the binding sites  
of the immobilized ligand.

Claim 5. (Amended) An assay method as claimed in [any one  
of claims 1 to 4] claim 1, wherein the presence or absence of a  
phosphatase-targeting toxin is determined.





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gold sol [labelled] labeled peptide heaptotoxin molecules capable of competitively inhibiting cyanobacterial toxins binding to said protein phosphatase.

Claim 19. (Amended) A kit as claimed in [any one of claims 15 to 17] claim 15, wherein said kit comprises magnetically displaceable polymer micro spheres having immobilized thereon a protein phosphatase;

gold sol [labelled] labeled okadaic acid molecules capable of competitively inhibiting algal toxins binding to said protein phosphatase.

Claim 20. (Amended) Use of the kit as claimed in [any one of claims 15 to 19] claim 15, for the determination of phosphatase-targeting toxins.

**IN THE ABSTRACT:**

A new Abstract is inserted.

Assay for phosphatase-targetting toxins

5 The present invention relates to an assay method  
for the detection of phosphatase-targeting toxins  
typically produced by microalgae such as for example  
cyanobacteria and dinoflagellates.

10 Dinoflagellates are typically unicellular,  
photosynthetic, bi-flagellated algae. Some of the  
marine dinoflagellates (e.g. *Prorocentrum sp.* and  
*Dinophysis sp.*) produce phosphatase-targeting toxins  
such as okadaic acid and dinophysis toxin, which cause  
gastrointestinal problems if ingested by humans. Such  
algae can thus be problematic if they contaminate the  
15 habitats of shellfish for consumption.

Cyanobacteria, which are often referred to as blue-  
green algae, are also photosynthetic organisms which are  
principally aquatic and inhabit coastal waters, open sea  
and oceans, rivers, lakes and ground water but may also  
20 be terrestrial and found in leaf litter and soil.

Many species and strains of cyanobacteria, in  
particular *Microcystis sp.*, *Aphanizomenon sp.*, *Anabena*  
*sp.*, *Nodularia sp.* and *Oscillatoria sp.*, produce toxins  
which if ingested by humans or other mammals, birds and  
25 even fish, can produce illness. Ingestion of such  
toxins occurs by two main routes, either by drinking  
contaminated water or by eating contaminated seafood.

Two particular types of toxins are produced by  
cyanobacteria and dinoflagellates. Neurotoxins, for  
30 example anatoxins and saxitoxins, cause paralysis in the  
victim and hence the condition often referred to as  
paralytic shellfish poisoning. Poisoning by such  
neurotoxins is rare but can prove to be fatal.

The other form of toxins inactivate protein  
35 phosphatase enzymes in the cells of the body by binding  
to the enzymes and affecting their ability to  
dephosphorylate protein substrates. These toxins are

- 2 -

relatively common, and some (such as the dinoflagellate toxins okadaic acid and dinophysis toxin) can cause nausea, vomiting and diarrhoea and hence the condition often referred to as diarrhoetic shellfish poisoning.

5 Some protein phosphatase-targeting toxins are tumour promoters and exposure to these toxins may lead to cancer. Others, such as the cyanobacterial toxins microcystin and nodularin are hepatotoxic and cause liver damage. The most prevalent of the phosphatase  
10 targeting toxins are microcystin, nodularin and okadaic acid.

The most common sources of dinoflagellate toxin poisoning are shellfish and fish liver, and the most common cause of cyanobacterial toxin poisoning is  
15 contaminated drinking and/or bathing water. Both cyanobacterial and dinoflagellate toxins may however be harboured in shellfish and in water. A particularly common source of algal toxin poisoning is mussels since they accumulate the toxins upon feeding on toxin-  
20 producing algae. Other shellfish, for example oysters, clams and scallops can also be affected.

Additionally, domestic water supplies, particularly if they originate from ground water, can become contaminated with cyanobacteria and thus provide a  
25 direct route for toxin ingestion.

There is some concern regarding consumption of algae and cyanobacteria as a high-protein health food and diet aid. There are no official guidelines for monitoring collected algae or cyanobacteria for  
30 contamination by toxin producing strains and the marketing of genera such as *Anabena* and *Aphanizomenon* is particularly worrying since a number of toxin producing strains may be found within them.

In addition to the short term discomfort, medical  
35 costs, commercial costs to the shellfish industry, loss of working hours etc. which result from exposure to

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ARTICLE 34

- 3 -

algal toxins, as mentioned above the phosphatase targeting toxins microcystin and nodularin have been found to be tumour promoters and it is believed that repeated exposure to such toxins at the clinical or sub-clinical level, particularly in combination with a high intake of alcohol or smoking may result in cancer, especially of the liver.

Presently, a number of different methods exist for the detection and quantitation of phosphatase targeting toxins, from algae and cyanobacteria. One standard method involves grinding mussels or other potential sources of the phosphatase targeting toxins and injecting an extract of the ground mussel tissue into mice. The presence and level of phosphatase-targeting toxin contamination is then determined in relation to mouse survival (Stabell et al. (1992), Food. Chem. Toxicol. 30(2): 139-44). Clearly, this is a time consuming, crude and expensive method of assessing food safety and quality control.

Another method for determining diarrheal shellfish poisons (DSP) (EP-A-554458 of Iatron Laboratories Inc) involves the use of a first and second antibody to the toxin in a conventional sandwich assay.

Another method involves measuring the reduction in enzymic activity of exogenously added phosphatase thus detecting the presence of phosphatase targeting toxins in the shellfish. Again this involves grinding mussels or other shellfish tissue, releasing endogenous phosphatases which interfere with the added phosphatase, compromising the sensitivity and accuracy of the test (Sim and Mudge (1994) in Detection Methods for Cyanobacterial Toxins Eds. Codd, Jeffries, Keevil and Potter, Royal Society of Chemistry, and US-A-5180665 of Charles Holmes.

A great need exists therefore for a quick, sensitive, and inexpensive assay or method to allow the qualitative and/or quantitative determination of the

ARTICLE 34

- 3a -

presence of phosphatase-targeting toxins, in particular algal and cyanobacterial phosphatase-targeting toxins, in water, shellfish and/or edible products of algae or cyanobacteria. In particular, there is a need for an assay method which is simple enough to be performed on

5

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5           Thus, according to a first aspect, the present invention provides an assay method for determining phosphatase targeting toxins which inhibit protein phosphatases comprising contacting a solid support having an immobilized ligand thereon with:

(ii) a non-immobilized ligand,

wherein said immobilized ligand is capable of generating directly or indirectly a detectable signal when uncomplexed, when complexed by said toxin, when complexed by a complex of said toxin and said non-immobilized ligand or when complexed by said non-immobilized ligand or said non-immobilized ligand is capable of generating a directly or indirectly detectable signal when uncomplexed or when complexed, separating a bound fraction from a non-bound fraction; and

35       directly or indirectly determining the non-immobilized ligand bound to the immobilized ligand (the bound fraction) or non-complexed in aqueous solution (the non-bound fraction);

- 5 -

wherein the application of (i) and (ii) to the solid support may be performed separately, sequentially or simultaneously and if separately or sequentially, they can be performed in either order.

5 Thus in one embodiment toxin determination may involve determination of the non-immobilized ligand which has failed to bind directly or indirectly to the immobilized ligand. Where the non-immobilized ligand competes for binding to the immobilized ligand with the  
10 toxin a high level of unbound ligand is indicative of a high toxin concentration. Where the non-immobilized ligand can complex toxin bound to the immobilized ligand then a high level of unbound ligand is indicative of a low level of toxin concentration.

15 In another embodiment, toxin determination involves determination of the non-immobilized ligand which has bound directly or indirectly to the immobilized ligand. Where toxin and non-immobilized ligand compete for binding to the immobilized ligand then a high level of  
20 bound ligand is indicative of a low level of toxin concentration. Where the non-immobilized ligand can complex toxin bound to the immobilized ligand then a high level of bound ligand is indicative of a high level of toxin concentration.

25 Preferably however the method of the invention involves a competitive binding assay for the detection of phosphatase-targeting toxins, in particular algal and cyanobacterial toxins, wherein toxin molecules present in a sample compete with the non-immobilized ligand for  
30 a limited number of binding sites of the immobilized ligand and any toxin present in said sample is determined relative to the extent of non-immobilized ligand bound to or not bound to the binding sites of the immobilized ligand.

35 As used herein, the terms "detecting" "determining" or "assessing" include both quantitation in the sense of obtaining an absolute value for the

- 6 -

amount or concentration of phosphatase-targeting toxins, present in the sample and also semi-quantitative and qualitative assessment or determination. An index, ratio, percentage or molar indication of the level or amount of toxin present may be determined or alternatively a simple indication of presence or absence of such toxins in the sample, may be obtained. In a preferred aspect of the invention a simple presence or absence or semi-quantitative determination of toxin presence is achieved. In this regard "absence" of toxin may mean that the toxin concentration is below the detection limit of the assay or is below a level deemed to be safe or tolerable.

The samples used in the assay method of the invention may be any sample suspected of exposure to phosphatase-targeting toxins, perhaps by exposure to phosphatase-targeting toxin producing microorganisms, for example water which may be sea water, fresh water, ground water, water taken from lakes, rivers, wells, streams, reservoirs, domestic water supplies or may be moisture extracted from shellfish for example by simple draining or extraction using a pipette or water in which shellfish have been allowed to soak or may be a foodstuff, food additive, nutritional supplement, alternative remedy or similar product which is produced by or from algae or cyanobacteria. Where shellfish contain free water (e.g. as in oysters), the assay may involve dipping an absorbent substrate (the solid support) into that water. Alternatively it may simply involve pressing an absorbent substrate against the damp flesh of the shellfish, e.g. after breaking on opening the shell.

In a preferred aspect of the invention the sample under investigation is surface or free moisture from shellfish.

All types of shellfish, for example scallops, prawns, mussels, and oysters are susceptible to the



- 7 -

assay method of the invention but in a preferred aspect, the shellfish are mussels. In another preferred aspect, the sample under investigation is water taken from the habitat in which such shellfish live and in a further preferred aspect, the sample is water taken from domestic water supplies.

The sample used for analysis may be used in an essentially untreated manner but may optionally be filtered by any known method or diluted by adding water, buffer or any other aqueous medium prior to analysis and may be stored or preserved for example by chilling or freezing prior to analysis.

Any toxin binding ligand may be used in the method of the invention as the immobilized or non-immobilized ligand for example antibodies, which may be polyclonal or monoclonal, or antibody fragments for example F(ab), F(ab')<sub>2</sub> or F(v) fragments. Such antibodies or antibody fragments may be monovalent or divalent and may be produced by hybridoma technology or be of synthetic origin, either as products of recombinant DNA technology or chemical synthesis. Single chain antibodies or other antibody derivatives or mimics could for example be used. The antibodies or antibody fragments may be directed or raised against any epitope, component or structure of the phosphatase-targeting toxins as appropriate. Alternatively, compounds with an affinity for the toxin for example a small organic molecule or peptide, e.g. an oligopeptide or polypeptide, capable of specifically binding the toxin for example a specific binder selected from a combinatorial chemistry or phage display library or a specifically binding sequence of DNA or RNA could be used.

Preferably however, the toxin binding ligand of the present invention is a protein phosphatase enzyme, and even more preferably the binding ligand protein phosphatase 2A (pp2A) is used in the assay method.

Likewise, the second ligand used in the method of

- 8 -

the invention may be any ligand which binds to the toxin either competitively or non competitively with the first ligand. Alternatively, the second ligand may be any ligand which competes with the toxin for binding to the first ligand. Preferably the first ligand is a toxin binding ligand, more preferably a protein phosphatase enzyme. One of the two ligands must be immobilized and the other must be non-immobilized and one of the ligands must be directly or indirectly detectable. In a preferred embodiment the non-immobilized ligand should meet the functional requirements that it competitively inhibits toxin binding to the immobilized ligand and can directly or indirectly produce a detectable signal, e.g. it may be a molecule which can be labelled using a direct or indirect signal forming moiety of any known form. Such ligands may likewise take the form of antibodies, which may be polyclonal or monoclonal, or antibody fragments for example F(ab), F(ab')<sub>2</sub> or F(b) fragments. Such antibodies or antibody fragments may be monovalent or divalent and may be produced by hybridoma technology or be of synthetic origin, either recombinant DNA technology or chemical synthesis. Single chain antibodies or other antibody derivatives or mimics and small organic molecules, peptides, oligopeptides and polypeptides selected from combinatorial or phage display libraries, could for example be used. The antibodies or antibody fragments may be directed or raised against any epitope, component or structure of the phosphatase-targeting toxin molecule or the ligand which binds the phosphatase targeting molecule as appropriate. Alternatively, compounds with an affinity for the toxin or for the ligand which binds the toxin, for example a small organic molecule or peptide, oligopeptide or polypeptide capable of specifically binding the toxin or the ligand which binds the toxin, for example a specific binder selected from a

- 9 -

combinatorial chemistry or phage display library, or a specifically binding sequence of DNA or RNA could be used.

5 The reporter moiety which one of the ligands will generally carry may be a binding site for a directly detectable moiety, e.g. a metal sol (e.g. gold sol), a chromophore or fluorophore (e.g. a cyanine, phthalocyanine, merocyanine, triphenylmethyl, equinane, etc. see Topics in Applied Chemistry, Infrared Absorbing  
10 Chromophores, edited by M. Matsuoka, Plenum Press, New York, NY, 1990, Topics in Applied Chemistry, The Chemistry and Application of Dyes, Waring et al. Plenum Press, New York, NY, 1990, and Handbook of Fluorescent Probes and Research Chemicals, Haugland, Molecular  
15 Probes Inc. 1996, a radiolabel, an enzyme, a magnetic particle, a turbidity inducing agent, etc., or it may already carry such a directly detectable moiety. Where the reporter moiety is carried by the immobilized ligand it will generally be a binding site for a directly  
20 detectable moiety which binding site is either activated, or more generally deactivated, when the ligand is complexed.

Preferably the reporter moiety is carried by the non-immobilized ligand.

25 In a preferred embodiment of the invention, the non-immobilized ligand is a labelled, e.g. enzyme or chromophore or fluorophore labelled peptide hepatotoxin, e.g. a hepatotoxin selected from nodularin, microcystin LC or microcystin YR or alternatively  
30 okadaic acid.

While labelling with radiolabels is possible, since the assay is primarily intended for on-site use by lay users, it is preferable to use reporter moieties that give a visible signal, e.g. chromophores, fluorophores,  
35 phosphorescent moieties, turbidity inducing agents, gas evolution inducing agents, etc.

Where the signal forming moiety is a material which

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- 10 -

binds to a binding site on one of the ligands, it will conveniently be contacted with the bound or unbound fraction, as appropriate, after separation of the bound and unbound fractions.

5 In general, where the signal is to be derived from the bound fraction, it will be preferable to rinse the substrate, e.g. with water, to flush away the unbound fraction before the ligand is detected or generated and detected.

10 Any species or strain of algae or cyanobacteria which produces phosphatase-targeting toxins may be subject to the present invention but it is particularly applicable to toxin producing strains of cyanobacteria for example *Microcystis aeruginosa*, *Anabena* species, 15 *Nodularia spurgana* and *Anabena flus-aquae* or algae. Thus for example the toxins microcystin-LR and microcystin-YR are produced by *Microcystis sp.*, the toxin nodularin is produced by *Nodularia sp.* and the toxin okadaic acid is produced by *Prorocentrum sp.*

20 The toxins subject to determination by the present method may likewise be any phosphatase-targeting toxin produced by algae or cyanobacteria, but in preferred aspects the peptide toxins are hepatotoxins (of which microcystin and nodularin are the most prevalent) or 25 okadaic acid.

Thus, in its most general sense, the method of the invention involves simply contacting a sample suspected of contamination with phosphatase-targeting toxins, with a toxin binding ligand and a reporter molecule capable 30 of competing with said toxin for the binding sites of the ligands either simultaneously, sequentially or separately in either order, the reporter molecule optionally being bound to the binding ligand prior to exposure to the sample under investigation, and 35 determining the reporter molecule which is either bound to the solid phase or free in solution.

The bound fraction may be separated from the unbound

- 11 -

5       faction prior to assessment of reporter by any suitable means, for example, precipitation, centrifugation, filtration, chromatographic means, capillary action or simply by draining. The solid phase may for example be in the form of a dipstick or a solid matrix in any known form for example polymeric or magnetic beads for example Dynabeads® (available from Dynal AS). In preferred embodiments of the present invention, the solid phase to which the toxin binding ligands are immobilised is in the form of Dynabeads®.

10       The reporter molecule may be assessed in either the bound or the non-bound fraction depending on the specific embodiment of the invention but preferably it is assessed in the bound fraction.

15       The immobilized ligand may be immobilised by any known means, for example by binding or coupling the ligand to any of the well known solid supports or matrices which are currently widely used or proposed for separation or immobilisation for example solid phases may take the form of particles, sheets, gels, filters, membranes, fibres or capillaries or microtitre strips, tubes or plates of wells etc. and conveniently may be made of glass, silica, latex, a polymeric material or magnetic beads. Techniques for binding the ligand to the solid support are well known in the art and widely described in the literature. In preferred embodiments of the present invention, the solid phase to which the phosphatase-targeting toxin binding ligands are immobilised is in the form of Dynabeads®.

25       The assay method of the present invention is advantageous in that it can be performed without the need of complex laboratory equipment and can be performed by the relatively non-skilled or non-skilled person. Hence, the assay method is suitable for use in the home, in shops or in the field and it can be performed quickly and easily without the need for intensive labour or hazardous chemicals.

- 12 -

Of particular advantage in the assay of the present invention is the very high degree of sensitivity which is of critical importance when analysing samples wherein the toxin is present at very low levels for example in the testing of drinking water or assessing possible pollution with phosphatase targeting toxins. Typically the assay is capable of detecting toxins in picomolar concentrations, e.g. as low as 10 pM. Conveniently the assay may be used to detect toxins in the 15 to 560 pM range.

A further advantage of the present assay relative to existing techniques is that the present assay is not affected by the presence of endogenous phosphatases which may be present in the samples under analysis, particularly, for example, if the samples are taken from shellfish.

In one embodiment of the present invention, a protein phosphatase is immobilised on a solid support, the immobilised phosphatase is contacted with the sample under investigation and any phosphatase-targeting toxin present in the sample binds to the immobilised phosphatase. A source of reporter molecules which compete with the toxin for phosphatase binding sites is added. The reporter molecules displace toxin molecules from the binding sites to a degree which depends upon the relative concentration of toxin molecules and reporter molecules. The degree of reporter molecule binding facilitates determination of toxin present in the sample under investigation. Preferred reporters/labels include radiolabels, chromophores (including fluorophores) and enzymes which give rise to chromogenic or fluorogenic products. Scintillation proximity labels and labels which give rise to a measurable change in light scattering are also to be considered.

In an alternative embodiment, solid support immobilised reporter-blocked phosphatase molecules are contacted with the sample under investigation and any

- 13 -

phosphatase-targeting toxins present in the sample compete with the phosphatase bound reporter molecules displacing them from the solid phase into the aqueous phase in a degree proportional to the amount of toxin present in the sample. The amount of reporter molecule which remains bound to the solid phase is then assessed to facilitate determination of toxin presence in the sample under investigation.

Viewed from a further aspect, the invention provides a kit for the detection of cyanobacterial or algal phosphatase-targeting toxins, according to the invention, said kit comprising:

a solid phase upon which is immobilised a ligand;  
a non-immobilized ligand, preferably in aqueous solution or complexed to the immobilized ligand; where neither of said immobilized and non-immobilized ligands includes a directly or indirectly detectable moiety, a reporter moiety capable of binding to one of said immobilized and non-immobilized ligands and generating a detectable signal, preferably said detectable moiety or signal being directly readable without laboratory equipment.

In one preferred embodiment, the kit of the present invention comprises:

a solid phase upon which is immobilized phosphatase-targeting toxin binding ligands;  
a reporter molecule capable of competitively inhibiting binding of phosphatase-targeting toxins to said toxin binding ligand and generating a signal readable without laboratory equipment.

An especially preferred embodiment of the kit of the invention comprises magnetically displaceable polymer micro spheres having immobilized thereon a protein phosphatase;

gold sol labelled peptide hepatotoxin molecules capable of competitively inhibiting cyanobacterial toxins binding to said protein phosphatase.

- 14 -

A further especially preferred embodiment of the kit of the invention comprises magnetically displaceable polymer micro spheres having immobilized thereon a protein phosphatase;

- 5 gold sol labelled okadaic acid molecules capable of competitively inhibiting algal toxins binding to said protein phosphatase.

- 10 In another preferred aspect, use of the kit involves dipping a porous cellulosic substrate on which a toxin binding ligand is immobilized and which is impregnated with a competitively binding, chromophore (or fluorophore etc) labelled ligand into a sample of water or shellfish fluid, allowing the saturated substrate to incubate for a pre-set period (either removed from the sample or in a pre-set volume of the sample), removing non-bound labelled ligand, e.g. by flushing the substrate with toxin-free water or by leaving the substrate to soak for a pre-set period in a pre-set volume of toxin free water, and inspecting the colour of the substrate or of the soaking water.
- 15 Desirably, the substrate is mounted on a support, preferably one marked with calibration colours to facilitate comparison of the substrate or soaking water colour to determine toxin concentration or to indicate whether toxin concentration is above or below one or more threshold values.
- 20
- 25

The invention will now be illustrated by the following non-limiting examples:

30 **Materials**

- Microcystin YR, Microcystin-LR, okadaic acid, nodularin, calyculin A and tautomycin are purchased from Calbiochem (San Diego, CA). Carrier-free  $\text{Na}^{125}\text{I}$  and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  is obtained from Amersham (Little Chalfont, UK). Albumin (RIA grade), ammonium acetate, Chloramine T, dimethyl sulfoxide (DMSO), dithioerythritol (DTE), EDTA, EGTA,
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- 15 -

glycerol, Hepes, histone II-AS, sodium metabisulfite and trypsin inhibitor (soybean) are purchased from Sigma (St Louis, MO). Acetonitrile and trifluoroacetic acid (TFA) are purchased from Rathburn (Walkerburn, Scotland).

- 5 Partially purified protein phosphatase 2A is either purchased from Upstate Biotechnology (Lake Placid, NY) or purified according to Resink et al. (Eur. J. Biochem. 133: 455-461 (1983)).

10 Iodination of microcystin-YR

Microcystin YR (10 µg) is iodinated with 1 mCi carrier-free Na<sup>125</sup>I (37 MBq) using chloramine T as described by Ciechanover et al., (PNAS 77: 1365-1368 (1980)).

- 15 Following the iodination reaction, iodide is separated from [<sup>125</sup>I]microcystin-YR using Sep-Pak® Plus cartridges (Waters, Milford, MA) according to the method of Runnegar et al. (Toxicon 24: 506-509 (1986)). The [<sup>125</sup>I]microcystin-YR is applied to a 3x250 mm Inertsil ODS-2 HPLC column from Chrompack (Raritan, NJ) and  
20 eluted with an acetonitrile gradient.

Competitive binding assay

- 25 The competitive binding assay is carried out in a volume of 0.5 ml buffered with 50 mM Hepes (pH 7.2), 1 mM EDTA, 0.3 mM EGTA, 1 mM DTE, 5 mM MnCl<sub>2</sub>, 0.5 mg ml<sup>-1</sup> BSA, and 0.2 mg ml<sup>-1</sup> trypsin inhibitor. Algal toxins diluted in 100% DMSO are added to the assay at 0-100 nM in a final  
30 concentration of 10% DMSO. [<sup>125</sup>I]microcystin-YR (1 Ci/13 ng) is added at 35 pM. Protein phosphatase 2A (30 pM) is added last, and the reaction mixture is incubated on ice overnight. [<sup>125</sup>I]microcystin-YR bound to protein phosphatase 2A is separated from free [<sup>125</sup>I]microcystin-YR by gel filtration using Sephadex® G-50 fine from  
35 Pharmacia (Uppsala, Sweden) in 0.7 x 15 cm columns from Bio-Rad (Hercules, CA). A 50 mM Hepes buffer (pH 7.2)

- 16 -

- with 1 mM EDTA and 0.3 mM EGTA is used in the separation which is done at 4°C. The fraction containing [<sup>125</sup>I]microcystin-YR which binds to protein phosphatase 2A is collected and the radioactivity is quantitated by scintillation counting. Nonspecific binding of [<sup>125</sup>I]microcystin-YR is detected in a control reaction where microcystin-LR is added at an excess (1 μM).

Example 1

- Protein phosphatase 2A is coupled to magnetic beads (directly to the beads or via biotinylation of the phosphatase). Immobilized protein phosphatase is then
- 5 mixed with sample and radiolabelled toxin (e.g. [<sup>125</sup>I]-microcystin-YR). The immobilized protein phosphatase is separated from the reaction mixture by magnetic force. Radioactivity associated with the protein phosphatase (magnetic bead) is detected by scintillation counting.
- 10 The amount of radiolabel associated with the protein phosphatase decreases as a function of phosphatase binding toxin in the sample.

Example 2

- 15 Protein phosphatase 2A is coupled to magnetic beads (directly to the beads or via biotinylation of the phosphatase). Immobilized protein phosphatase is then mixed with sample and toxin coupled to colored beads. The immobilized protein phosphatase is separated from
- 20 the reaction mixture by magnetic force. Colored beads associated with the protein phosphatase (magnetic beads) are evaluated by eye or by a low magnification microscope (e.g. Nikon TMS). The amount of colored beads associated with the protein phosphatase (magnetic
- 25 beads) decreases as a function of phosphatase binding toxin in the sample.

Example 3

- Protein phosphatase 2A is coupled to magnetic beads (directly to the beads or via biotinylation of the phosphatase). Immobilized protein phosphatase is then
- 30 mixed with sample and toxin immobilized on beads carrying an immobilized enzyme. The enzyme is capable of producing a detectable product (colored or
- 35 fluorescent) upon appropriate incubation with a chromogenic or fluorogenic substrate. The immobilized protein phosphatase is separated from the reaction

- 18 -

- mixture by magnetic force. Color or fluorescence associated with the protein phosphatase (magnetic beads) is measured by spectroscopy or fluorimetry, respectively. The amount of color/fluorescence associated with the magnetic beads decreases as a function of phosphatase binding toxin in the sample.

#### Example 4

Scintillation Proximity Assay:

- Protein phosphatase is biotinylated and immobilized to wells precoated with streptavidin and a scintillant (e.g. FlashPlate PLUS Streptavidin SMP103 supplied by NEN). The sample and [ $^{125}$ I]microcystin-YR are added to the wells. The amount of [ $^{125}$ I]microcystin-YR bound to the immobilized protein phosphatase is detected by scintillation counting.

#### Example 5

- Inhibition of binding of [ $^{125}$ I]-microcystin-YR to protein phosphatase 2A in the presence of various toxins

Compound tested <sup>1</sup>	IC <sub>50</sub> <sup>2</sup> (pM)
nodularin	15
microcystin-LR	17
microcystin-YR	75
okadaic acid	100
calyculin A	251
tautomycin	562

- <sup>1</sup> The compounds tested were incubated with [ $^{125}$ I]-microcystin-YR and protein phosphatase 2A as described above.

<sup>2</sup> The IC<sub>50</sub> value represents the concentration needed to obtain a 50% inhibition of [ $^{125}$ I]-microcystin-YR

- 19 -

binding to protein phosphatase 2A. These values were determined according to Fig. 3. The data represent an average of at least 3 separate experiments.

# 5 Example 6

## Effect of exogenous compounds on the competitive binding assay as compared to the protein phosphatase assay

10	Compound tested <sup>1</sup>	% activity <sup>2</sup>	
		Competitive binding assay	Protein phosphatase assay
15	2 mM ATP	103.3 ± 0.2	9.8 ± 3.4
	0.5 mM ATP	101.6 ± 1.7	29.8 ± 5.6
	0.05 mM NaPPi	101.4 ± 4.1	14.2 ± 1.2
	50 mM NaF	101.5 ± 1.9	7.7 ± 1.4
	5 mM NaF	102.0 ± 3.3	62.6 ± 0.4
20	1 mg/ml caseine	98.6 ± 4.5	3.4 ± 0.2
	0.02 mg/ml caseine	98.9 ± 6.1	33.3 ± 4.9
	5 mg/ml histone 2A	91.9 ± 1.8	1.4 ± 0.1
	0.002 mg/ml histone	95.2 ± 4.7	63.6 ± 4.0
	0.5 M NaCl	41.2 ± 0.7	44.4 ± 1.6
25	seawater	34.8 ± 0.4	ND
	10% seawater	87.3 ± 0.4	ND
	10% DMSO	72.8 ± 2.3	97.9 ± 3.3
	10% MeOH	73.9 ± 0.5	87.4 ± 4.1
	10% acetonitrile	90.4 ± 5.4	88.2 ± 2.7
30	0.4% Triton X-100	122.3 ± 1.0	60.2 ± 5.7
	0.4% Nonidet P-40	106.0 ± 2.0	61.1 ± 1.3
	0.4% CHAPS	90.9 ± 9.9	138.0 ± 34.4

<sup>1</sup> Protein phosphatase 2A was preincubated with the compounds dissolved in 50 mM Hepes (pH 7.2) or with buffer alone (control) for 30 minutes on ice. Phosphatase activity was measured by dephosphorylation of phosphohistone as described.

The % activity is relative to the control reaction.

<sup>2</sup> The activity in the competitive binding assay represents the ability of protein phosphatase 2A to

- 20 -

bind [ $^{125}$ I]microcystin-YR in the presence of the exogenous compound dissolved in buffer relative to buffer alone. The data represents an average of at least three separate experiments  $\pm$  SEM.

5

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Example 7Sensitivity of the binding assay for nodularin and microcystin-LRinhibition of [<sup>125</sup>I]microcystin-YR binding (%)<sup>1</sup>

toxin	(M)	milliQ water	drinking water	sea water	sea water, 1/10 <sup>2</sup>
nodularin	1E-10	88.37 ± 0.31	88.75 ± 0.16	72.18 ± 0.82	67.24 ± 0.66
	5E-11	36.37 ± 2.28	36.12 ± 1.04	48.47 ± 0.79	52.98 ± 1.98
microcystin-LR	1E-10	84.91 ± 0.42	86.97 ± 1.12	73.31 ± 1.20	46.41 ± 5.97
	5E-11	13.87 ± 3.16	12.85 ± 0.88	49.34 ± 3.82	38.61 ± 1.49

<sup>1</sup> Nodularin and microcystin-LR were dissolved in MilliQ, drinking, or sea water at the concentration shown. Aliquots of 300 µl of these solutions were tested for their ability to compete with [<sup>125</sup>I]microcystin-YR for the binding of protein phosphatase 2A as described above.

<sup>2</sup> Sea water diluted 1/10 in milliQ water.

The data is presented as the average ± SEM.

Example 9

Okadaic acid equivalents in shellfish extracts as  
determined by HPLC analysis and by the protein  
phosphatase binding assay

Extract <sup>1</sup>	OA equivs. by HPLC analysis <sup>2</sup>	OA equivs by binding assay <sup>3</sup>	
		( $\mu\text{g/g}$ hepatopancreas)	(nM)
1	0	0	85
2	0	0	45
3	0	0	70
4	4	2480	2100
5	1.2	748	755
6	0.8	496	805

- <sup>1</sup> The extracts were made from hepatopancreas of mussels collected along the Norwegian coast.
- <sup>2</sup> The extracts were analyzed for okadaic acid equivalents by HPLC.
- <sup>3</sup> The extracts were diluted in 100% DMSO and tested for their ability to compete with [<sup>125</sup>I]microcystin-YR for binding to protein phosphatase 2A using the binding assay as described above. The concentration of okadaic acid equivalents were determined by comparing the data to standard curves of okadaic acid dissolved in 100% DMSO.

Example 9Attached Diagrams

- Fig. 1 of the attached diagram is a schematic diagram of the competitive binding assay for the detection of protein phosphatase binding toxins.



- 23 -

Protein phosphatase 2A is incubated with [ $^{125}$ I]microcystin-YR and another toxin directed towards protein phosphatase 2A. The toxin competes with the [ $^{125}$ I]microcystin-YR for binding to the phosphatase.

5 Addition of a large amount of toxin results in a reduced binding of [ $^{125}$ I]microcystin-YR to the phosphatase and vice versa. After binding equilibrium is reached, the [ $^{125}$ I]microcystin-YR bound to protein phosphatase 2A is separated from free [ $^{125}$ I]microcystin-YR by gel

10 filtration chromatography. The fraction containing [ $^{125}$ I]microcystin-YR bound to the phosphatase is collected and the amount of radioactivity determined by scintillation counting.

15 Fig. 2 of the attached diagrams shows the effect of increasing amounts of different algal toxins on binding of [ $^{125}$ I]microcystin-YR to protein phosphatase 2A.

Protein phosphatase 2A (30 pM) was incubated in the presence 35pM [ $^{125}$ I]microcystin-YR (1 Ci/13 ng) and 0-100 nM of different algal toxins indicated in the figure. The [ $^{125}$ I]microcystin-YR bound to protein phosphatase 2A was isolated by gel filtration chromatography and the radioactivity determined by scintillation counting.

25 Each curve represents the average of at least 3 separate experiments.

Fig. 3 of the attached diagrams shows the  $IC_{50}$  for microcystin-LR binding in the competitive binding assay.

30 Binding of [ $^{125}$ I]microcystin-YR to protein phosphatase 2A was plotted as the ratio between unbound [ $^{125}$ I]microcystin-YR (Co-Cx) and bound [ $^{125}$ I]microcystin-YR (Cx) against the concentration of microcystin-LR. Co

35 represents the amount of bound [ $^{125}$ I]microcystin-YR in the absence of microcystin-LR, and Cx represents the amount of bound [ $^{125}$ I]microcystin-YR in the presence of

- 24 -

various concentrations of microcystin-LR.

Fig. 4 of the attached diagrams illustrates the stability of the [ $^{125}$ I]microcystin-YR bound to protein phosphatase 2A in the presence of excess microcystin LR.

Protein phosphatase 2A (1 nM) was incubated in the presence of [ $^{125}$ I]microcystin-YR (100 pM) for 1 hour. Microcystin-LR (2  $\mu$ M) was added to the reaction mixture at time 0. The amount of [ $^{125}$ I]microcystin-YR bound to protein phosphatase 2A was determined for the indicated timepoints by gel filtration and scintillation counting as described. The curve represents an average of 4 separate experiments.

- 25 -

## Claims:

1. An assay method for determining phosphatase targeting toxins which inhibit protein phosphatases
- 5 comprising contacting a solid support having an immobilized ligand immobilized thereon with:
- (i) a sample suspected of being contaminated with toxin and
- (ii) a non-immobilized ligand,
- 10 wherein said immobilized ligand is capable of binding to at least one of said toxins, to said non-immobilized ligand or to complexes of said toxin and said non-immobilized ligand, and said non-immobilized ligand is capable of binding to at least one of said
- 15 immobilized ligand, to said toxin or to complexes of said toxin and said immobilized ligand whereby the proportion of said immobilized ligand bound by said toxin, said non-immobilized ligand or complexes of said toxin and said non-immobilized ligand is dependent on
- 20 the toxin content of said sample and wherein the immobilized and/or non-immobilized toxin binding ligand is a protein phosphatase enzyme and
- wherein said immobilized ligand is capable of generating directly or indirectly detectable signal when
- 25 uncomplexed, when complexed by said toxin, when complexed by a complex of said toxin and said non-immobilized ligand or when complexed by said non-immobilized ligand or said non-immobilized ligand is capable of generating a directly or indirectly
- 30 detectable signal when uncomplexed or when complexed, separating a bound fraction from a non-bound fraction; and
- directly or indirectly determining the non-immobilized ligand bound to the immobilized ligand (the
- 35 bound fraction) or non-complexed in aqueous solution (the non-bound fraction);
- wherein the application of (i) and (ii) to the

- 26 -

solid support may be performed separately, sequentially or simultaneously and if separately or sequentially, they can be performed in either order.

- 5        2.    An assay method as claimed in claim 1 wherein said phosphatase-targeting toxin is produced by algae or by cyanobacteria.
- 10       3.    An assay method as claimed in either of claims 1 and 2 wherein the toxin to be determined is a heptatoxin or okadaic acid.
- 15       4.    An assay method as claimed in any one of claims 1 to 3 wherein toxin molecules present in the sample compete with the non-immobilized ligand for a limited number of binding sites of the immobilized ligand and any toxin present in said sample is determined relative to the extent of non-immobilized ligand bound to or not bound to the binding sites of the immobilized ligand.
- 20       5.    An assay method as claimed in any one of claims 1 to 4 wherein the presence or absence of a phosphatase-targeting toxin is determined.
- 25       6.    An assay method as claimed in any one of claims 1 to 5 wherein the sample under investigation is surface or free moisture from shellfish, or water taken from the habitat in which such shellfish live, or water taken from domestic water supplies.
- 30       7.    An assay method as claimed in any one of claims 1 to 6 wherein the immobilized or non-immobilized ligand is an antibody or antibody fragment.
- 35       8.    An assay method as claimed in any one of claims 1 to 7 wherein the protein phosphatase enzyme is the binding ligand protein phosphatase 2A.

- 27 -

9. An assay method as claimed in any one of claims 1 to 8 wherein either the immobilised or non-immobilised ligands carries a reporter moiety.
10. An assay method as claimed in claim 9 wherein the non-immobilized ligand carries a reporter moiety.
11. An assay method as claimed in claim 10 wherein the non-immobilized ligand is a labelled peptide hepatotoxin or labelled okadaic acid.
12. An assay method as claimed in claim 11 wherein the hepatotoxin is selected from nodularin, microcystin LC or microcystin YR.
13. An assay method as claimed in any one of claims 1 to 12 wherein the solid support is a dipstick or solid matrix.
14. An assay method as claimed in claim 13 wherein the solid matrix is polymeric or magnetic beads.
15. A kit for the detection of phosphatase-targeting toxins which inhibit protein phosphatases, said kit comprising:
- a solid phase upon which is immobilised a ligand;
  - a non-immobilized ligand, preferably in aqueous solution or complexed to the immobilized ligand;
  - and wherein the immobilized and/or non-immobilized ligand is a protein phosphatase enzyme;
- where neither of said immobilized and non-immobilized ligands includes a directly or indirectly detectable moiety, a reporter moiety capable of binding to one of said immobilized and non-immobilized ligands and generating a detectable signal, preferably said detectable moiety or signal being directly readable without laboratory equipment.

- 28 -

16. A kit as claimed in claim 15 wherein said phosphatase-targeting toxin is produced by algae or by cyanobacteria.

- 5 17. A kit as claimed in either of claims 15 and 16 wherein said kit comprises:

a solid phase upon which is immobilized a protein phosphatase enzyme as a toxin binding ligand;

- 10 a reporter molecule capable of competitively inhibiting binding of phosphatase-targeting toxins to said toxin binding ligand and generating a signal readable without laboratory equipment.

- 15 18. A kit as claimed in any one of claims 15 to 17 wherein said kit comprises magnetically displaceable polymer micro spheres having immobilized thereon a protein phosphatase;

- 20 gold sol labelled peptide hepatotoxin molecules capable of competitively inhibiting cyanobacterial toxins binding to said protein phosphatase.

- 25 19. A kit as claimed in any one of claims 15 to 17 wherein said kit comprises magnetically displaceable polymer micro spheres having immobilized thereon a protein phosphatase;

gold sol labelled okadaic acid molecules capable of competitively inhibiting algal toxins binding to said protein phosphatase.

- 30 20. Use of the kit as claimed in any one of claims 15 to 19 for the determination of phosphatase-targeting toxins.

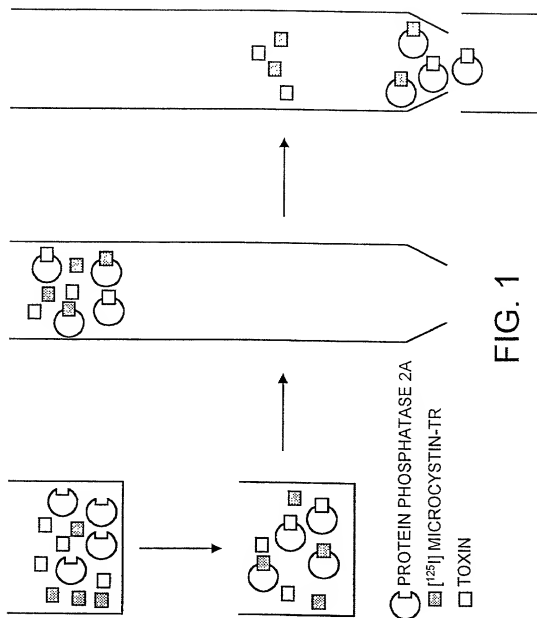


FIG. 1

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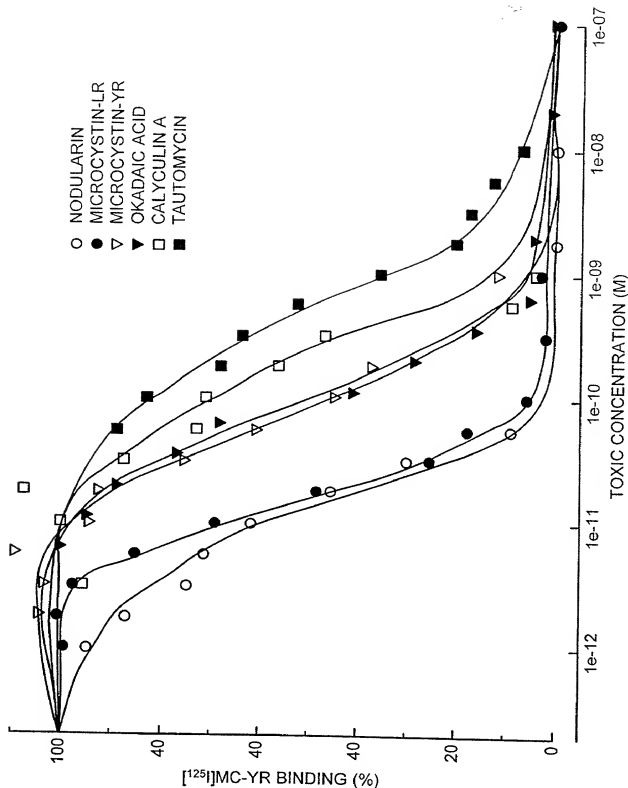


FIG. 2



3 / 4

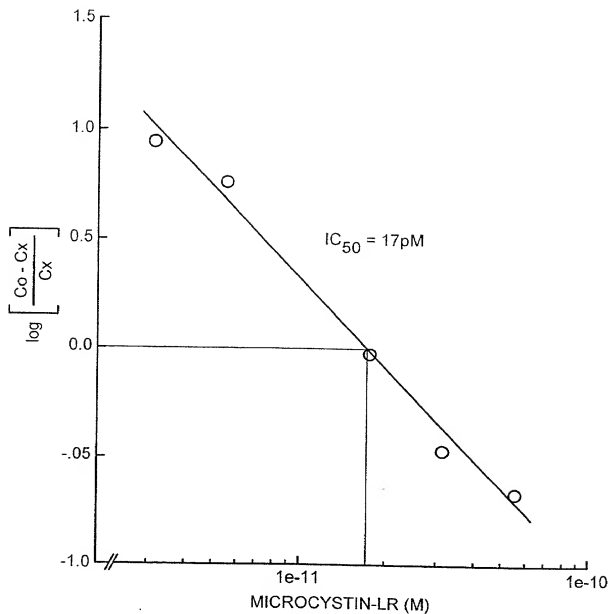


FIG. 3

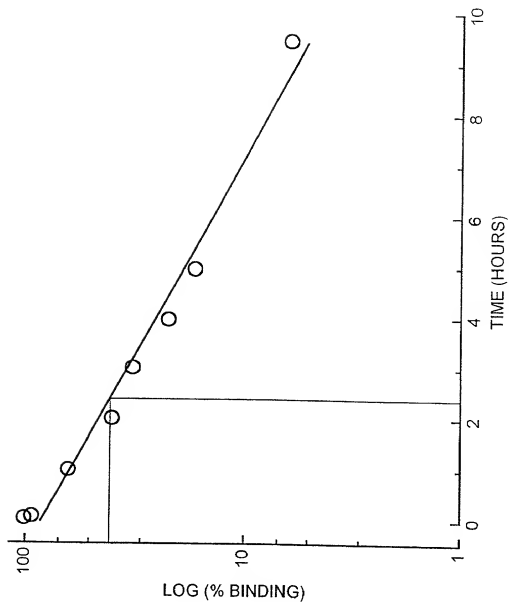


FIG. 4

DECLARATION AND POWER OF ATTORNEY

As the below named inventors hereby declare that our residence, post office address and citizenships are as stated below next to our names: that we believe we are the original and joint inventors of the subject matter claimed and for which a patent is sought in the application entitled:

ASSAY FOR PHOSPHATASE-TARGETING TOXINS

which application is:

☐ the attached application  
(for original application)

☒ Application No. \_\_\_\_\_  
filed May 4, 2001, and amended on \_\_\_\_\_  
May 4, 2001  
(for declaration not accompanying application)

that we have reviewed and understand the contents of the specification of the above-identified application, including the claims, as amended by any amendment referred to above; that we acknowledge our duty to disclose information of which we are aware which is material to the patentability of this application under 37 C.F.R. § 1.56, that we hereby claim priority benefits under Title 35, United States Code § 119, § 172 or § 365 of any provisional application or foreign application(s) for patent or inventor's certificate listed below and have also identified on said list any foreign application for patent or inventor's certificate on this invention having a filing date before that of any foreign application on which priority is claimed:

Application Number	Country	Filing Date	Priority Claimed
GB 99/03756	PCT	November 11, 1999	YES
9824772.9	Great Britain	November 11, 1998	YES

We hereby claim the benefit of Title 35, United States Code § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in a listed prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, We acknowledge our duty to disclose any information material to the patentability of this application under 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status
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We hereby appoint John H. Mion, Reg. No. 18,879; Thomas J. Macpeak, Reg. No. 19,292; Robert J. Seas, Jr., Reg. No. 21,092; Darryl Mexic, Reg. No. 23,063; Robert V. Sloan, Reg. No. 22,775; Peter D. Olexy, Reg. No. 24,513; J. Frank Osha, Reg. No. 24,625; Waddell A. Biggart, Reg. No. 24,861; Louis Gubinsky, Reg. No. 24,835; Neil B. Siegel, Reg. No. 25,200; David J. Cushing, Reg. No. 28,703; John R. Inge, Reg. No. 26,916; Joseph J. Ruch, Jr., Reg. No. 26,577; Sheldon I. Landsman, Reg. No. 25,430; Richard C. Turner, Reg. No. 29,710; Howard L. Bernstein, Reg. No. 25,665; Alan J. Kasper, Reg. No. 25,426; Kenneth J. Burchfiel, Reg. No. 31,333; Gordon Kit, Reg. No. 30,764; Susan J. Mack, Reg. No. 30,951; Frank L. Bernstein, Reg. No. 31,484; Mark Boland, Reg. No. 32,197; William H. Mandir, Reg. No. 32,156; Scott M. Daniels, Reg. No. 32,562; Brian W. Hannon, Reg. No. 32,778; Abraham J. Rosner, Reg. No. 33,276; Bruce E. Kramer, Reg. No. 33,725; Paul F. Neils, Reg. No. 33,102; Brett S. Sylvester, Reg. No. 32,765; Robert M. Masters, Reg. No. 35,603 and George F. Lehnigk, Reg. No. 36,359, our attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC, 2100 Pennsylvania Avenue, N.W., Washington, D.C. 20037-3213.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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